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The use of Epstein-Barr virus transformation for the production of human monoclonal antibodies

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Summary. Current aspects of the production of murine and human monoclonal antibodies are reviewed. The use of murine monoclonal antibodies in the treatment of a variety of human tumors has met with limited success due to reactions to the xenogeneic antibodies. Human antibodies offer certain potential advantages for therapeutic use and because of this interest, techniques including hybridization and EBV transformation are being developed for their production. We contrast these two methods and emphasize the special relationship that exists between EBV and human B lymphocytes. Results from this and other laboratories suggest that transformation alone or in combination with hybridization will be a viable method for producing human antibodies with useful specificities.

Key words: Epstein-Barr virus, Transformation, Lymphoblastoid cell lines, Monoclonal antibodies

Introduction

The production of murine monoclonal antibodies by lymphocyte-myeloma hybridomas (Kohler and Milstein 1975) has had a revolutionary impact on biological research. By immunizing mice, removing the immune spleen cells, and fusing them with mouse myeloma cells, many research groups have produced cloned antibody-producing tumor cells ("hybridomas") of

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predefined specificity (reviews: Kennett 1979; Siniscalco 1979; Williams 1979; Kazmar and Fathman 1980; Kennett et al. 1980; Milstein 1980; Yelton and Scharff 1980; Reading 1982a; Kozbor and Roder 1983). Each of these separate clones produces antibody to a single determinant of the immunogen. By analysis of the individual specificities of the hybridomas, the polyclonal antibody response can be segregated into specific and nonspecific clones. In this manner, exquisitely specific immunological reagents can be produced. Since the clones which remain stable for antibody production and for proliferation can be grown in culture indefinitely and as ascites tumors in mice, a virtually unlimited supply of these reagents can be prepared, and identical antibodies can be utilized around the world.

Murine monoclonal antibodies

The procedures involved in murine monoclonal antibody production are described in Fig. 1. We have recently reviewed the use of in vitro immunization for monoclonal antibody production (Reading 1982a, 1983) and presented examples of its use for the production of monoclonal antibodies reactive with glycoproteins (Reading 1981), conserved antigens such as calmodulin (Pardue et al. 1981), and murine (Miner et al. 1981) and human tumor cells (Reading 1982b; Dicke et al. 1982).

The technology of mouse monoclonal antibody production has now reached the stage where anti-tumor reagents have been developed and have begun to be used in the diagnosis and treatment of human cancers (Reviews: Ritz and Schlossman 1982; Levy and Miller 1983; Poynton and Reading 1983; Davis and Rao 1983). Murine monoclonal antibodies have been used in initial clinical therapeutic trials in leukemias and lymphomas (Ritz and Schlossman 1982; Miller et al. 1982; Dillman et al. 1982b; Levy and Miller 1983; Poynton et al. 1983) and gastrointestinal tumors (Sears et al. 1982). Radiolabeled anti-tumor monoclonal antibodies have been used to image CEA-positive carcinomas (Mach et al. 1981; Wahl et al. 1983; Smedley et al. 1983; Goldenberg 1983), ovarian, breast, thyroid and gastrointestinal tumors (Epenetos et al. 1982; Farrands et al. 1982; Lumbroso et al. 1983; Chatal et al. 1983), and melanomas (Larson et al. 1983).

These initial studies have been encouraging, but there have also been some problems associated with the use of murine monoclonal antibodies in humans. Sears and co-workers (1982) have found in a phase-I clinical trial of monoclonal antibodies directed against human gastrointestinal tumors that after a single injection three of the four patients treated developed antibodies against the mouse protein. One patient developed an anaphylactic response after repeated injections, which correlated with a significantly increased rate of clearance of the mouse immunoglobulin from the patient's serum. In patients receiving radiolabeled monoclonal antibodies to image melanoma (Larson et al. 1983) all of the patients tested developed anti-mouse antibodies. In these patients decreased tumor localization and

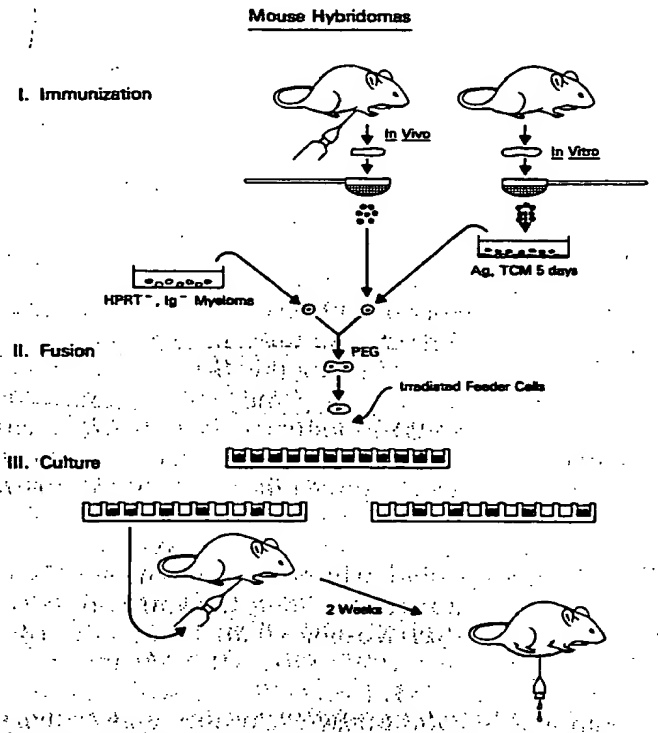


Fig. 1. Schematic representation for the production of mouse monoclonal antibodies. Immune splenocytes are fused with mouse myeloma cells that are enzyme deficient. The hybrids are grown in HAT medium, which selects only for hybrids between splenocytes and myeloma cells. Cultures are tested for specific antibody production and subcultured. After a period of growth the cells are cloned in 96-well plates using irradiated feeder cells. Large amounts of antibody can be produced by ascites tumors of the hybridomas in mice. See text for further details.

increased clearance rate and localization in the liver were observed with subsequent injections of the same monoclonal antibody. Other studies have also indicated that mouse immunoglobulins are immunogenic in humans (Nadler et al. 1980; Ritz et al. 1981; Miller et al. 1981; Cosimi et al. 1981), and that anti-mouse antibodies may contribute to the failure of therapy in some cases (Dillman et al. 1982a; Levy and Miller 1983). Results such as these indicate that mouse monoclonal antibodies may be of limited use in the long-term management of cancer.

Another difficulty arises in the development of these murine monoclonal antibodies. Since mice preferentially recognize human determinants as foreign, a majority of hybrids derived from mice immunized with human material will make antibodies reactive with common human antigens, rather than with tumor-associated antigens. A great amount of screening and characterization is necessary to find antibodies that react with the tumor cells but have limited reactivity with normal tissues.

Producti n of human antibodies

Several difficulties might be overcome by the use of human monoclonal antibodies. Presumably tumor-bearing humans only respond to those tumor associated antigens that are altered from "self". In addition, human antibodies would present fewer antigenic determinants against which patients could respond (allogeneic and idiotypic determinants, instead of all xenogeneic determinants). Development of human monoclonal antibodies would allow the production of antibodies on a large scale to antigenic determinants that are poorly or not immunogenic in mice. For example, Crawford and co-workers (1983a) produced human antibodies to blood group Rh_D, which alone among the human blood group antigens is nonimmunogenic in mice. Furthermore, this field of research may help delineate which antigenic structures on pathogenic microorganisms and tumor cells are relevant to human immunity. The development of human monoclonal antibodies requires the immortalization of human B cells, either by hybridization with a tumor cell line or by transformation of the B lymphocyte directly.

Fusion of human B cells with human and murine myelomas has led tc the production of human-human hybridomas (Bloom and Nakamura 1974; Croce et al. 1980; Olsson and Kaplan 1980; Osband et al. 1981; Mills et al 1982; Eisenbarth et al. 1982; Kozbor et al. 1982; Edward et al. 1982 Chiorazzi et al. 1982; Handley et al. 1981, 1982; Shoenfeld et al. 1982; Sikora et al. 1983; Cote et al. 1983; Kozbor and Roder 1983; Houghton et al 1983) and human-mouse hybridomas (Schwaber and Cohen 1973, 1974 Schwaber 1975, 1977; Levy and Dilley 1978; Schlom et al. 1980; Nowinsk et al. 1980; Wunderlich et al. 1981; Teramoto et al. 1982; Lane et al. 1982 Kozbor et al. 1982; Cote et al. 1983), which secrete human antibodies Human cell lines suitable for fusion with human immune B lymphocyte have been studied (Kozbor and Roder 1983; Abrams et al. 1983). One o these lines (GM 1500 6TG-A1 2), a myeloma cell line derived from a patient with multiple myeloma, was selected for resistance to 6-thioguanin (6-TG). This cell line has been used successfully to produce human-huma hybridomas by hybridization with peripheral blood mononuclear cell (PBMC) from a patient with subacute sclerosing panencephalitis. The hyb ridomas selected secreted human IgM specific for the measles virus nu cleocapsids (Croce et al. 1980). A second line (U266-AR_1) was obtained b 8-azaguanine (8-Ag) selection of an EBV transformed human lymphc blastoid cell line and has been used to create human-human hybridomas b fusion with spleen cells from a patient previously sensitized to 2,4-dinitrc chlorobenzene. This line was also used to produce hybridomas with bot human spleen cells and PBMC after in vitro priming with sheep red bloo cells (SRBC) (Olsson and Kaplan 1980). Another drug-selected huma myeloma cell line LICR-LON-HMy2 has been used to produce humar human hybridomas by fusion with intratumoral lymphocytes from a pa tient with a malignant glioma. Purified human monoclonal antibody wa radiolabeled and used to image recurrent glioma in the same patient (Phi

lips et al. 1983). The authors did not report any untoward response to the autologous human antibody. This antibody was also used for serotherapy with no apparent therapeutic effect (Watson et al. 1983a). Human monoclonal autoantibodies have been produced by fusion of human myeloma cells with peripheral blood lymphocytes from patients with systemic lupus erythematosus (Cambon-de-Mouzon and Olsson 1983). Technical aspects and limitations of this human hybridoma technique have been reviewed (Olsson and Kaplan 1983; Olsson 1983). The technology of human-human hybrid production is not yet developed to the same extent as that of mouse-mouse hybridomas.

Another approach to human monoclonal antibody production has been to make human-mouse hybridomas. Schlom and co-workers (Schlom et al. 1980; Wunderlich et al. 1981; Teramoto et al. 1982) fused cells from draining lymph nodes from breast cancer patients with murine myeloma NS-1 cells to obtain human-mouse hybridomas. Tumor-reactive human monoclonal antibodies have also been obtained by fusion of lymph node lymphocytes from lung cancer patients with both rat and mouse myeloma cells (Sikora and Wright 1981). Nine of the cell lines obtained, produced antibodies unreactive with normal lung cells from the same patient. Interspecies hybrids may lack the stability of intraspecies hybrids, although Schlom and co-workers (1980) were able to obtain some stable mouse-human hybrids. The major significance of this approach is that they made hybrids from immune B lymphocytes from cancer patients, and that this led to the production of antibodies with selective reactivity towards the tumor.

A disadvantage of producing monoclonal antibodies from hybridomas is that the technology of lymphocyte-myeloma hybridoma production is quite complicated. The myeloma cell line must be maintained in culture and have drug resistance markers to eliminate the growth of unfused cells (Kit et al. 1963; Littlefield 1963). These hybrids must be grown in medium containing aminopterin, hypoxanthine, thymidine, and glycine (HAT medium; Szybalski et al. 1962; Littlefield 1964) after fusion. The myelomas used for hybridization should not produce heavy or light immunoglobulin chains. If immunoglobulin-producing cell lines (Kohler and Milstein 1975; Margulies et al. 1976a) are used to form hybridomas, the two sets of heavy and light chains can combine intracellularly to form mixed immunoglobulin molecules (Cotton and Milstein 1973; Margulies et al. 1976b). The use of immunoglobulin nonsecretors (Kohler et al. 1976), which synthesize only immunoglobulin light chains may lead to immunoglobulin molecules in the hybrids that contain mixtures of light chains. Immunoglobulin "nonproducers", which synthesize neither heavy nor light chains, have been described in the murine system (Trowbridge 1978; Shulman et al. 1978; Kearney et al. 1979). Nonproducers, such as the Sp2 cell line, occasionally reexpress the myeloma light chain (D. Katz, personal communication). To date, nonproducer human myeloma fusion partners have not been reported. We have derived a nonproducer human myeloma, MDA 200, which we have selected for resistance to 8-azaguanine, but are only currently investigating its potential as a fusion partner.

Stability of immunoglobulin secretion by the hybridomas is anoth major concern, particularly with interspecies hybrids. The stability of tl hybridomas is in part dependent upon the myeloma cell line used f fusion. The genetic stability of hybridomas produced from reselected ce hybrids, such as the Sp2 and F_0 (Shulman et al. 1978; Frazekas de S Groth et al. 1980), may be inferior to that of hybridomas produced fro myelomas. In addition to these difficulties, the frequency of hybridom formation is low (less than one hybrid from 10^4 splenocytes).

EBV transformation

Direct immortalization of B cells could conceivably be accomplished by number of methods, including chemical mutagenesis, transfection wit oncogenic DNA, the use of growth factors, irradiation, or viral infectio While unlimited growth is important, it is equally important that immunc globulin production be stimulated and sustained. Transformation usin viruses seems to be the most promising avenue and has received the mo attention. Collins and co-workers (1974) and Strosberg and co-worker (1974) reported the use of SV_{40} to transform rabbit splenocytes and pro duce antibody-secreting continuous cell lines. This transformation appear to be a rare event, and we and others have been unsuccessful in using SV to transform mouse or human B cells (Baumal 1971; J. T. Hutchins an C. L. Reading, unpubl.).

A more popular agent has been Epstein-Barr virus (EBV), a herpes vi rus that has been used to selectively transform B lymphocytes from a varie ty of primates including man (Miller and Lipman 1973). It is the causativ agent of infectious mononucleosis and is suspected in the etiology o Burkitt's lymphoma, nasopharyngeal carcinoma (Klein 1979), and th X-linked lymphoproliferative syndrome (Purtilo et al. 1981). Cell lines of a B cell nature have been derived from the peripheral blood of infectiou mononucleosis (Pope 1967) and Burkitt's lymphoma patients (Pulvertaf 1964). In vitro infection of B lymphocytes by EBV can result in both trans formation (Katsuki et al. 1977) and polyclonal activation of immunoglo bulin secretion (Leibold et al. 1975), and, as such, offers a method for th long-term production of human immunoglobulins. Human antibodies reac tive with haptens and bacterial polysaccharides (Steinitz et al. 1977, 1978 Kozbor et al. 1979), tetanus toxoid (Zurawski et al. 1978a, b; Kozbor and Roder 1981), immunoglobulins (Steinitz et al. 1980); blood group antigens (Koskimies 1980; Boyston et al. 1980; Crawford et al. 1983a), virus nu cleoprotein (Crawford et al. 1983b), chlamydial antigen (Rosen et al. 1983), viral antigens (Seigneurin et al. 1983), malarial antigens (Lundgren et al. 1983), and various tumor associated antigens that will be discussed below, have been obtained after EBV transformation of immune B lym phocytes.

EBV is perhaps the most efficient transforming virus known, with B cell transformation rates estimated to be as high as 10% to 30% (Henderson et al. 1977; Miller 1980). A variety of assay systems have been used to meas-

are the efficiency of transformation, including growth on feeder cells and growth in semisolid agarose. The variety of transformation rates reported can be attributed to differences in the sensitivity of the assay employed, skill in enriching for B cells, differences among donors, existence of prior immunity to EBV, the source of B cells (e.g. cord blood or peripheral blood), variations in culture conditions, and differences among virus preparations. Generally, EBV transformation does not confer to cells the ability to grow well in soft agar, with values of one in 10^4 to 10^3 being reported (Yamamoto and Hinuma 1976; Sugden and Mark 1977; Brown and Miller 1982). The highest rates of transformation have been the result of using suspension cultures with various types of cells as feeder layers (Zerbini and Ernberg 1983). A viral marker, which correlates with infection and transformation, the Epstein-Barr nuclear antigen (EBNA) (Leibold et al. 1975; Einhorn and Ernberg 1978), is found in 10% to 30% of B cells three days after in vitro infection (Bird et al. 1981a) and in virtually all transformed cells. Recently, however, Zerbini and Ernberg (1983) have compared the efficiency of infection of cells from human cord blood with the subsequent ability of the infected cells to grow and found that 19% to 97% of the B cells were infected (EBNA positive) and that 60% of the EBNA positive cells were able to grow in suspension. Variability was found to be due to differences between donors and batches of virus. By comparison, they found that only 1.4% to 3.7% of $EBNA^+$ cells could grow in soft agar. It has also been shown that phytohemagglutinin and lipopolysaccharide can enhance transformation (Henderson et al. 1977). The authors speculated that this might be due to enhancement of EBV DNA replication or enhancement of early virus-cell interaction.

The transformation of B cells can be inhibited by a subpopulation of T cells both in vitro and in vivo (Svedmyr and Jondal 1975; Thorley-Lawson et al. 1977; Haynes et al. 1979; Moss et al. 1979; Thorley-Lawson 1980). Tosato and co-workers (1982) have shown that infection of lymphocytes from EBV seropositive individuals results in the generation of suppressor cells after 10–12 days in culture that inhibit any further activation by either EBV or pokeweed mitogen (PWM). The inhibitory T cell population was never seen in seronegative individuals or in cord blood. It has been implied that the suppressive effect of T cells can be at least partially overcome by using higher titers of EBV (Thorley-Lawson et al. 1977). Furthermore, cyclosporine and hydrocortisone have been used to ablate the T cell suppression during the first two weeks of culture (Haynes et al. 1979; Palacios 1981; Bird et al. 1981b; Lundgren et al. 1983).

EBV also acts as a polyclonal activator of immunoglobulin production, with an increase in all classes of immunoglobulin seen within seven days of its addition to a culture of leukocytes (Rosen et al. 1977). In patients with active infectious mononucleosis there is an increase in the circulating levels of IgM, IgG, and IgA (Sutton et al. 1973). It has been reported that in culture there is no isotype switching induced by EBV (Brown and Miller 1982; Yarochoan et al. 1983). An exception to this was recently reported by Stein and co-workers (1983), who noted that a very small proportion of cells

switched from IgM to IgA. They speculated that this represents a population of cells preprogrammed to make this switch and, therefore, did not switch as a result of EBV infection.

Stein and co-workers (1983), Yarochoan and co-workers (1983) and Martinez-Maya and Britton (1983) have studied the frequency of B cells that can be stimulated to produce immunoglobulin using limiting dilution analysis. Two of these groups (Yarochoan et al. 1983; Martinez-Maya and Britton 1983) obtained similiar results, in that two weeks after infection only a small fraction of B cells were stimulated to secrete immunoglobulin. Furthermore, this was apparently a distinct population from that stimulated by PWM. Stein and co-workers, on the other hand, with various differences in the experimental design, found that after 4–6 weeks in culture, 8% of B cells were secreting immunoglobulin. Apparently, there was no preference for a particular isotype, rather the isotype pattern reflected the tissue from which the B cells were derived. It is difficult to compare experiments of this type from different laboratories, but the latter results indicate that the number of cells producing immunoglobulin may be comparable to the number expressing EBNA.

It is now accepted with some confidence that B cells are the exclusive lymphoid target of EBV infection (Schneider and Zurhauser 1975), and that virtually all B cells possess receptors for EBV (Greaves et al. 1975). It is not known, however, whether the stage of the B cell is a factor in transformation. There is some indication that perhaps EBV acts most effectively on resting B cells. As mentioned above, EBV and PWM activate separate populations of B cells (Martinez-Maya and Britton 1983). PWM is known to be a T cell-dependent B cell activator that is active on more activated B cells, which do not express the receptor for mouse red blood cells (MRBC), lack surface IgD, and bear surface IgG (Ault and Towle 1981; Kuritani and Cooper 1982). The surface markers present on EBV-sensitive B cells have not been well characterized, but Fong and co-workers (1983) have shown that a MRBC-positive fraction was activated by EBV to produce IgM anti-IgG autoantibodies. Although the MRBC-negative fraction produced IgM, it had little anti-IgG activity. The MRBC-negative fraction could however, be induced to produce IgM anti-IgG when stimulated with PWM. Additionally, B cells from umbilical cord blood are sensitive to EBV but relatively resistant to PWM, even when supplemented with adult T lymphocytes (Tosato et al. 1980). There is, furthermore, evidence that more activated B cells have fewer receptors for EBV (Jondal and Klein 1973; Chang et al. 1976; Tsukuda et al. 1982). Apparently, plasma cells lack EBV receptors entirely since Nilsson (1971) was unable to establish EBV positive lines from myeloma cells. At variance with these conclusions is the work of Steel and co-workers (1977), who showed that the IgM-bearing B cells are preferentially transformed in cord blood. Since cord blood produces antibodies primarily of the IgM type, their conclusion was that EBV transforms cells already secreting antibody, and, thus it is a more activated cell which is transformed. An alternative interpretation might be that the IgG- and IgA-bearing lymphocytes in cord blood are deficient in

me way (a notion that has some support, Gathings et al. 1981), and that conclusions can be reached concerning the level of activation of this nsformed IgM-bearing lymphocyte, since both activated and resting rms are present.

Luzzati and co-workers (1977) have used EBV in human in vitro immunizations with SRBC and found that EBV acts to stimulate specific antigen responses. Significantly, they found that UV irradiation of the virus d not negate the effect, implying that a membrane-binding event might rve to trigger the B cell or helper T cell recognizing viral antigens. Similly, Chang and Spina (1976) found that heat-inactivated virus stimulated e incorporation of tritiated thymidine into lymphocytes of seronegative nors, although they did not determine whether the stimulated cells were or T cells. Presumably, this could be a T cell response to viral antigens. contrast, Bird and co-workers (1979) discovered that inactivated EBV as ineffective in inducing polyclonal activation of human blood lymphotes as measured by a plaque assay. It seems likely that EBV may affect a ilture of leukocytes in more than one way: firstly, it is an activator of B lls via an antigen-independent receptor, and secondly, it presents a umber of viral antigens to both B and T cell antigen receptors, resulting a conventional immune response. In other systems viral antigens have en shown to provide helper determinants for otherwise weak immunoens (Bromberg et al. 1982).

Early reports indicated that only very low levels of antibody were produced by lymphoblastoid cell lines (Zurawski et al. 1978a, b; Kozbor and oder 1981). Recent advances, such as elimination of T cells, which can ppress antibody production (Haynes et al. 1979; Johnsen et al. 1979; ostato et al. 1979, 1982), and antigen preselection and cloning techniques Zurawski et al. 1978a,b; Steinitz et al. 1977, 1978, 1980; Kozbor et al. 979, Kozbor and Roder 1981; Koskimies 1980; Boylston et al. 1980) have d to production of 10 μg/ml of specific antibody (Steinitz et al. 1977, 978, 1980; Kozbor et al. 1979) and higher (Crawford et al. 1983b; M. ongnecker, personal communication), levels which are competitive with hose obtained from hybridomas (Reading 1982a; Schlom et al. 1980; Wunderlich et al. 1981; Teramoto et al. 1982). In an analysis of the immunoglobulins produced by lymphocyte lines established using EBV, Baumal nd co-workers (1971) determined that 5% to 30% of newly synthesized roteins are immunoglobulins, with most being fully assembled.

Preselection

Clearly, enrichment for the B cells with the desired binding site specificity would make the transformation and screening processes more efficient, as well as remove potential problems due to T and NK cells. Preselection of B cells specific for certain haptens or immunoglobulins by rosetting with appropriately derivatized red blood cells or by adherence to immobilized antigen has resulted in an enrichment in the numer of cells that secrete the desired antibody. Enrichment for antigen-specific B cells also allows the

use of nonimmune individuals as sources of B cells for transformati (Winger et al. 1983). However, as Rosen and co-workers (1983) ha pointed out, selection by rosetting enriches surface Ig-positive cells a these are not necessarily the subpopulation most highly secretory or m favorably transformed by EBV.

Another issue that should be considered is the choice of tissue as source of B cells. Certainly, human blood is more readily obtained th samples of other immune compartments, so most B cells may be deriv from peripheral blood out of necessity. However, when possible, ce should be obtained from those organs that might be rich in antigen-prim B cells. When tumor-reactive antibodies are sought, lymphocytes should obtained from the tumor tissue itself or from draining lymph nodes. B ce reactive with other antigens to which the donor has been exposed mi best be obtained from the bone marrow (Fauci and Pratt 1977; Fong et 1982). Seigneurin and co-workers (1983) have been able to establish sta (2-year) cell lines from the bone marrow of Herpes Simplex virus seropc tive persons which produce high levels (15 $\mu g/10^6$ cells per 24 h) of antil dy specific for HSV glycoprotein D. Interestingly, they report that t were unable to derive such a line from peripheral blood of the same p son. Furthermore, with certain antigens it may be possible to increase number of susceptible B cells by boosting with antigens either in v (Steinitz et al. 1977) or perhaps in vitro (Lundgren et al. 1983), althoug the latter case it has not been shown if the antigen acts specifically nonspecifically.

Production of tumor-reactive-antibodies

EBV has been used to transform B cells from patients with various ma nancies in order to produce cell lines making antibodies to tumor assoc ed antigens (Irie et al. 1981, 1982; Hirohashi et al. 1983; Watson et 1983b; Reading et al. 1983). We have also established lines from parti purified B-cells from lymph nodes of breast cancer patients which w transformed after preselection on irradiated breast tumor cells (Fig. These lines produce antibodies reactive with established breast tumor li (Cailleau et al. 1978), but not with normal human fibroblasts by enzy linked immunosorbent assays (ELISA); and reactive with the autolog breast tumor tissue, but not with adjacent uninvolved breast tissue (Fig. These lines were unstable and stopped producing antibodies after t months. Irie and co-workers (1982) have produced two stable B cell li that secrete antibodies reactive with an oncofetal antigen associated w human melanomas. The stable lines were the result of transforming c from peripheral blood leukocytes of 232 melanoma patients. Part of difficulty encountered by these investigators might be that the B cells re tive with tumor-associated antigens are only present in low numbers in p ipheral blood. By using B lymphocytes derived from tumor tissue as t gets, Watson and colleagues (1983) have derived several lines from t patients which produce antibodies specific for malignant melanoma ce

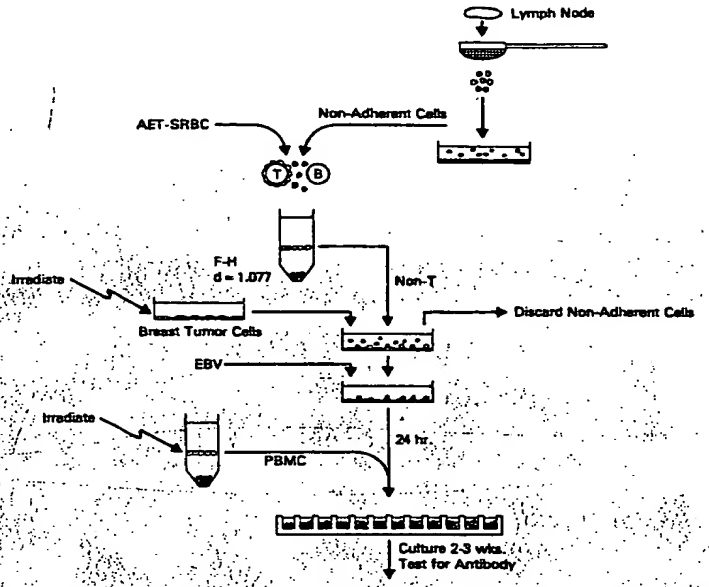
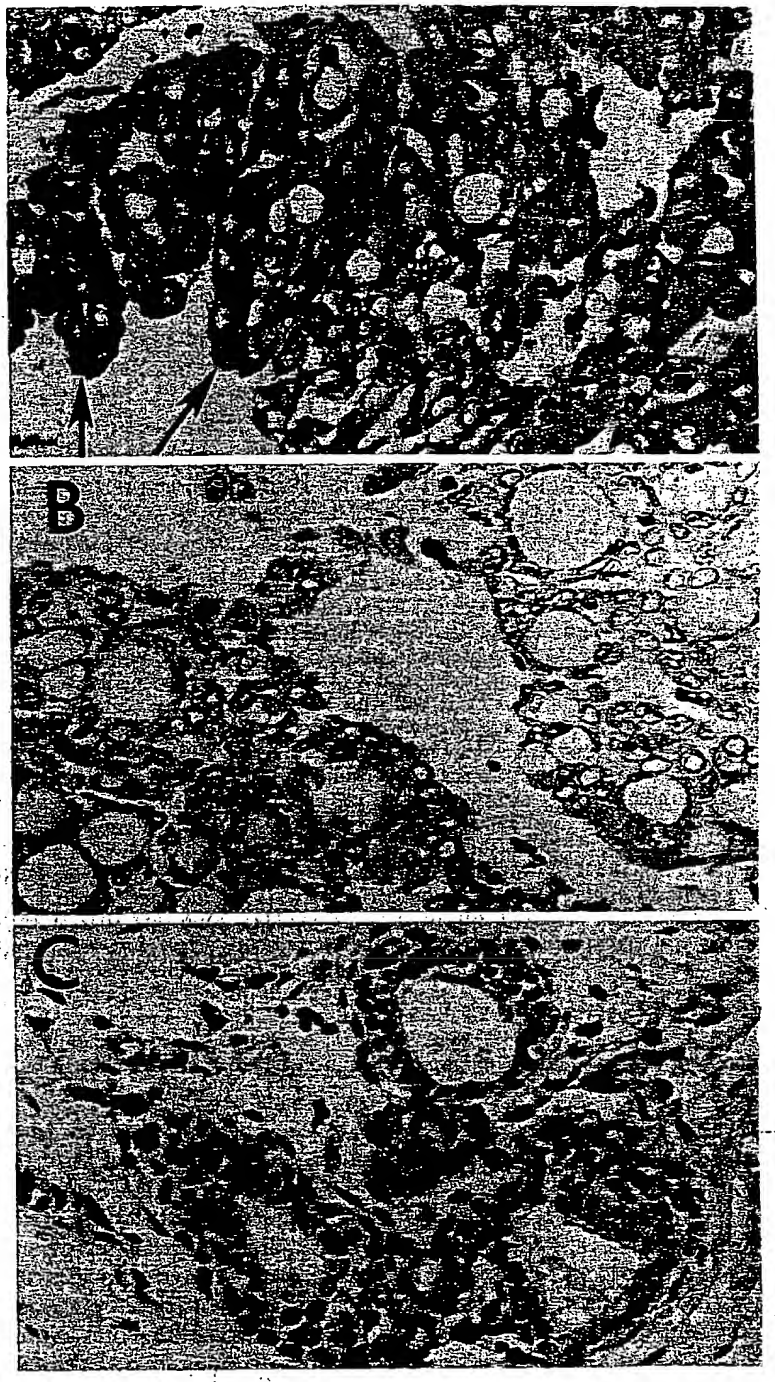


Fig. 2. Axillary lymph node tissue was obtained from a patient undergoing a modified radical mastectomy for malignant carcinoma of the breast. The cells were dissociated by pressing the tissue through a 50-mesh stainless steel screen with the rubber-tipped plunger from a disposable 12 ml syringe into RPMI 1640 medium containing 20% fetal bovine serum (FBS). The cells were pipeted 10 times to obtain a single cell suspension, washed once by centrifugation, and resuspended in 30 ml of fresh RPMI 1640 with 20% FBS. Adherent cells were removed by incubation in a 75 cm^2 tissue culture flask (Falcon) for 18 h. The nonadherent cells were removed, washed by centrifugation, resuspended in RPMI + 20% FBS at 10^7 cells/ml.

T lymphocytes were removed by rosette formation with s-(2-aminoethyl)isothiouronium bromide hydrobromide-treated sheep red blood cells (AET-SRBC) (Madsen et al. 1980), followed by separation on a Ficol-Hypaque (F-H, d = 1.077) interface (Verma et al. 1980). The interface cells, which are enriched for B lymphocytes, were washed twice by centrifugation, resuspended in 10 ml RPMI 1640 with 20% FBS, and incubated in a 75 cm^2 tissue culture flask containing confluent, adherent, ^{137}Ce-irradiated (4500 rad) human metastastic breast cancer cells from the MDA-MB 231 line (Cailleau et al. 1978) (231). After one hour at 37°, the nonadherent cells were removed and transfered to irradiated cells from metastatic breast cancer cell in MDA-MB 435s (435s). The cells remaining adherent to 231 cells were incubated in fresh RPMI 1640 with 20% FBS (4 ml) and 1 ml of medium containing EBV that had been concentrated 20-fold by ultracentrifugation of the supernatant fluid from a culture of the marmoset lymphoblastoid cell line MCU-V (the MCU-V cells were kindly provided by Dr. Zurawski from the Centocor Corporation). The lymphocytes nonadherent to 435s cells were discarded, and the adherent cells were incubated with EBV as above. After 24 hours, 2×10^7 irradiated (4500 rad) peripheral blood leukocytes (PBL) from a healthy volunteer were added to each flask and the cells were pipeted to dislodge them from the adherent layer. The dislodged cells were diluted and pipeted into 16-mm wells (Flow Labs).

They also mention that they were unsuccessful in establishing lines which produced antibodies specific for melanoma cells from the peripheral blood of 43 melanoma patients. It would appear from these results that the development of lines producing antibody specific for tumor-associated antigens is more efficient if the lymphocytes are derived from the tumor or its draining lymph nodes.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Unresolved difficulties

One of the major problems associated with the use of EBV for the production of human monoclonal antibodies is the eventual cessation or decrease in already low antibody production. In view of the work of many of the authors cited in this review, our own observations, and of the established mechanisms of B cell interaction with EBV, it is our opinion that to achieve the desired specificity in a monoclonal antibody the following steps should be taken:

a) Removal of T cells and macrophages, which enriches for B cells and lessens the likelihood of supression. However, there is the possibility an adherent subpopulation of cells exists that should accompany the B cells to stabilize them in culture (Schneider and Zurthausen 1975; Gergely and Ernberg 1977). The use of cyclosporine to eliminate T cell suppression of B cell growth (Bird et al. 1981b; Lundgren et al. 1983) may represent an alternative to B cell purification.

b) Enrichment for antigen-specific B cells by using immune individuals, tissues relatively rich in specific B cells, rosetting, panning, or antigen stimulation in vivo or in vitro. A question we and others are now asking is whether there is a certain subpopulation of B cells that is preferentially transformed.

c) Prompt cloning after infection, avoids high cell densities that may inhibit immunoglobulin production (Winger et al. 1983). Since transformation efficiency also includes plating efficiency, one must optimize culture conditions to optimize transformation efficiency. Certain B cell lines are able to respond to T-cell factors by producing increased amounts of immunoglobulins (Muraguchi et al. 1981; Teranishi et al. 1982). In that regard, it might be best use irradiated peripheral blood cells as feeder layers, since certain T cell-help is radiation resistant while suppressors and cytotoxic T cells are sensitive.

d) Fusion with an appropriate human myeloma cell line to rescue or potentiate antibody production.

Fig. 3A–C. Photomicrographs of immunoperoxidase-stained mastectomy specimen from a patient with ductal carcinoma of the breast. Paraffin-embedded tissue sections (4 μm thick) were digested with 0.1% trypsin (Fisher Scientific Co., Fair Lawn, N.J.) for 1 h at 37°C, washed with 0.05 M tris-buffered saline (TBS), and incubated either with control medium with serum or the MDA 151 antibody-containing medium diluted 1:10 with TBS. After 1 h the slides were washed twice for 10 min with TBS and incubated with horseradish peroxidase-conjugated IgG fraction of goat anti-human immunoglobulins (Meloy, Springfield, Va.) diluted 1:20 with TBS. After 30 min the slides were washed with TBS, and the substrate (0.1 ml of 3 amino-9-ethylcarbazole in N,N-dimethylformamide and 0.1 ml of 0.3% H_2O_2 diluted in 2 ml of 0.1 M acetate buffer, pH 5.2, Accurate Chemical & Scientific Co., Westbury, N.Y.) was added. The enzyme reaction was terminated after 45 min, the slides were washed and stained with Meyer's hematoxylin. A Photomicrograph of a ductal carcinoma showing positive cytoplasmic immunoreaction with MDA 151 (arrows). B Medium control of the corresponding region from a serial section demonstrating negative staining. C Uninvolved breast ducts adjacent to the tumor showing negative staining with MDA 151. (Magnification 250X).

Growth of cells as ascites tumors in nude mice has been a method f achieving large quantities of mouse monoclonal antibodies. Howev failure to be tumorigenic in nude mice has long been a hallmark of hum (non-Burkitt's Lymphoma) EBV-carrying lymphoblastoid lines (Nilss 1979). We have attempted unsuccessfully to grow our human lymph blastoid cell line in mineral oil-primed nude mice. Recent success wi growing human hybridomas in nude mice (V. Zurawski, personal comm nication) may or may not be translated to human lymphoblastoid cell lin and hybrids with human myelomas produced from them. An alternative the production of large amounts of antibody by growth of ascites tumors mice has been described by Frazekas de St. Groth (1983). A cytostat w constructed that allowed automated production of murine monoclonal a tibodies. This technology may also prove feasible for large-scale hum monoclonal antibody production.

The absence of EBV will have to be verified before clinical use of t antibodies produced by these cells is contemplated. Although the vast m jority of human B cells transformed with EBV are virus nonproducers small fraction may become lytic, releasing infective virions (Hampar 197 Selection for virus-nonproducer cell lines and biochemical purification the antibody may overcome contamination of the antibody preparations EBV. The same concern holds for the human and mouse antibodies p duced by hybridomas (Olson and Kaplan 1980; Bartal et al. 1982; We 1982). Recently, Crawford and co-workers (1983c) have minimized th concerns by suggesting that filtration, DNase treatment, and affinity pu fication of the preparation will be sufficient precaution against administ ing EBV or DNA fragments with the antibody. While the use of monoc nal antibodies produced in this way to treat human disease must await f ther progress, they should prove immediately useful for in vitro diagno procedures.

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